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The *Autographa californica* multiple nucleopolyhedrovirus *lef-5* gene is required for productive infection

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ABSTRACT

To examine the role of the AcMNPV *lef-5* gene in the context of the infection cycle, we generated an AcMNPV *lef-5* knockout virus (vAc^{lef5ko}) and a complementing cell line that supports viral replication. We examined AcMNPV DNA replication, early and late gene expression, and production of infectious viral progeny in the absence of *lef-5*. While early gene expression and DNA replication were not reduced by the *lef-5* knockout, expression of a late reporter was disrupted and representative late transcripts were dramatically reduced. Progeny virus production was not detected after transfection of Sf9 cells with the *lef-5* knockout bacmid, but was rescued by insertion of an *egfp*- or *myc*-tagged *lef-5* gene into the vAc^{lef5ko} genome. An *egfp*-tagged *lef-5* gene from *Se*MNPV was used to generate a stable Sf9 cell line that supported replication of the vAc^{lef5ko} virus. The LEF-5 protein was also found to co-localize with IE-1 in infected cell nuclei.

Introduction

The baculovirus *Autographa californica* Multiple Nucleopolyhedrovirus (AcMNPV) has a genome of approximately 134 kbp and encodes an estimated 155 genes (Ayres et al., 1994; Rohrmann, 2008; Theilmann et al., 2005). Gene expression can be divided into three major phases: early, late, and very late, and these phases appear to be regulated primarily at the level of transcription. Early phase genes are necessary for DNA replication and the subsequent transcriptional cascade. Viral structural proteins are generally expressed in the late phase from late promoters. While baculovirus early promoters are recognized by host cell RNA polymerase II and resemble host RNA polymerase II promoters, late promoters are transcribed by a virus-specific late RNA polymerase (Passarelli and Guarino, 2007; Rohrmann, 2008). Late promoters are compact and contain a highly conserved TAAG motif at the transcription start site. Inhibitors of DNA replication (such as aphidicolin) also inhibit late gene transcription, suggesting that viral DNA replication is required for late transcription. A group of 19 baculovirus genes capable of reconstituting late gene transcription was identified by transient expression assays (reviewed in (Lu and Miller, 1995; Rapp et al., 1998)). These genes were termed late expression factor or "*lef*" genes (Passarelli and Miller, 1993a). At least 10 *lef* genes are involved in, or are important for viral DNA replication, and these include *lef-1*, *lef-2*, *lef-3*, *lef-7*, *lef-11*, *ie-1*, *ie-2*, *p143*, *DNApol* and *p35* (Kool et al., 1994; Lin and Blissard, 2002b). A powerful tool for evaluating the functions and requirements for *lef* genes is the use of bacmids containing knockouts of *lef* genes in the AcMNPV genome. Using gene knockouts in the AcMNPV genome, *lef-2*, *lef-3*, *lef-11*, *ie-1*, *DNApol*, and *p143* were identified as essential for viral DNA replication in the context of the viral genome (Bideshi and Federici, 2000; Knebel-Moersdorf et al., 2006; Lin and Blissard, 2002b; Stewart et al., 2005; Vanarsdall et al., 2005; Wu et al., 2010; Yu and Carstens, 2010). Approximately 9 *lef* genes (*lef-4*, *lef-5*, *lef-6*, *lef-8*, *lef-9*, *lef-10*, *lef-12*, *p47* and *pp31*) are thought to be involved more directly in baculovirus late gene transcription (Lu and Miller, 1995; Rohrmann, 2008). Biochemical data indicate that four of these genes (*lef-4*, *lef-8*, *lef-9* and *p47*) encode subunits of the viral late RNA polymerase complex (Guarino et al., 1998). LEF-4 was previously identified as an mRNA capping enzyme and is essential for viral replication (Gross and Shuman, 1998; Guarino et al., 1998; Knebel-Moersdorf et al., 2006). LEF-6 and PP31 are not essential for viral replication but appear to either accelerate late transcription or increase most viral transcript levels, respectively (Lin and Blissard, 2002a; Yamagishi et al., 2007). While transient assays represent a powerful approach for studies of *lef* gene functions, studies of *lef* gene knockouts have refined and extended the roles of some *lef*

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genes, particularly regarding their requirements for DNA replication and/or late transcription in the context of the infection cycle (Guarino et al., 2002b; Li et al., 1999; Lin and Blissard, 2002b; Rapp et al., 1998).

The *lef-5* gene of AcMNPV was initially identified as an AcMNPV gene that is required for transient expression by a late promoter–reporter gene construct (p*VP39-cat*) in a screen for late expression factors (Passarelli and Miller, 1993b). The *lef-5* gene is a core baculovirus gene, found in all baculoviruses sequenced to date (Herniou and Jehle, 2007; Herniou et al., 2003). Prior attempts to knock out the *lef-5* gene in *Bombyx mori* nucleopolyhedrovirus (BmNPV) were unsuccessful and knockout viruses could not be obtained, leading to the supposition that *lef-5* may be essential for BmNPV replication (Gomi et al., 1997). In prior studies of the AcMNPV LEF-5 protein, a C-terminal domain with similarity to the zinc ribbon domain of RNA polymerase II elongation factor IIS (TFIIS) was identified and was found to self-interact in yeast two-hybrid and GST pull-down assays (Harwood et al., 1998). However, studies using truncated forms of *lef-5* indicate that LEF-5 activity in the late transient expression assay is partially retained even in the absence of the Zn ribbon domain. Although LEF-5 has sequence similarities with an elongation factor, results from *in vitro* experiments suggested that LEF-5 functions as an initiation factor (Guarino et al., 2002a). In recent studies, it was also demonstrated that the LEF-5 protein from SeMNPV can a substitute for the AcMNPV LEF-5 protein in transient late expression assays (Berretta and Passarelli, 2006).

Although transient late expression studies suggest that most *lef* genes have essential functions in the AcMNPV infection cycle, analyses of knockout mutations indicate that some *lef* genes (*e.g. lef-6*, *pp31)* are dispensable for viral replication in the context of the infected cell (Lin and Blissard, 2002a; Yamagishi et al., 2007). In the current study, we asked whether LEF-5 was required for AcMNPV replication. To examine its overall role in the viral infection cycle, we generated and analyzed a *lef-5* knockout bacmid (bAc^{lef5ko}). We found that bAc^{lef5ko} was unable to productively replicate in Sf9 cells but was rescued by reinserting a *lef-5* gene. We also generated a cell line expressing an EGFP-tagged LEF-5 from *Spodoptera exigua* MNPV (SeLEF5EGFP) and found that the cell line was capable of rescuing infectivity of bAc^{lef5ko}, the *lef-5* knockout bacmid. Using the AcMNPV *lef-5* knockout virus (vAc^{lef5ko}) generated from the SeLEF5EGFP-expressing cell line, we examined the effect of the *lef-5* knockout by analyzing viral DNA replication, late gene expression, and viral replication.

Results

Generation of an AcMNPV lef-5 knockout bacmid and rescue with AcMNPV and SeMNPV lef-5 constructs

To examine the role of LEF-5 in the context of an AcMNPV infection, we generated an AcMNPV virus containing a knockout in the *lef-5* gene. A *lef-5* knockout was generated in AcMNPV bacmid bMON14272, as described earlier (Datsenko and Wanner, 2000; Yamagishi et al., 2007). The central portion (314 bp) of the *lef-5* ORF was replaced with a cassette containing a late promoter-driven reporter gene (*p6.9-GUS*) and a chloramphenicol resistance gene (*cat*). The resulting bacmid was named as (bAc^{lef5ko}). The *lef-5* ORF overlaps two adjacent genes (*38K* and *p6.9*) (Fig. 1A). To avoid disrupting expression of those adjacent genes, portions of the 5′ and 3′ ends of the *lef-5* ORF were retained in the knockout bacmid construct and the central portion of the *lef-5* ORF was deleted. The resulting bacmid (bAc^{lef5ko}) retained 264 bp (AcMNPV nt 85918–86181) from the 5′ end of the *lef-5* ORF, and 220 bp (nt 86496–86715) from the 3′ end of the *lef-5* ORF. Deletion of *lef-5 ORF* sequences and insertion of the *cat-GUS* cassette were confirmed by PCR and sequencing.

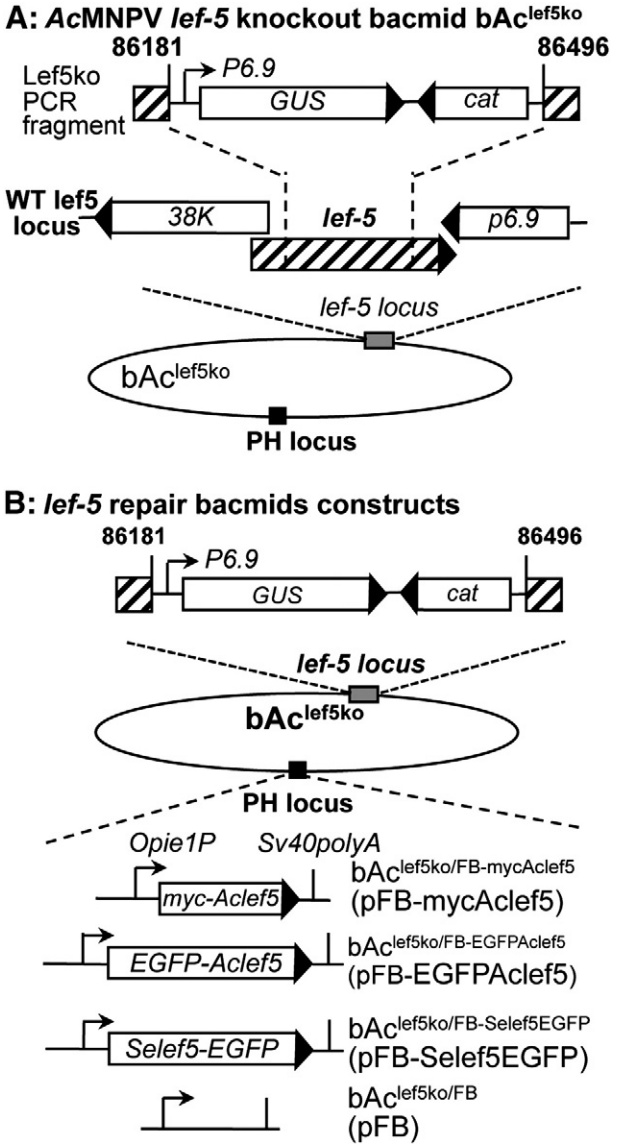

Fig. 1. Generation of AcMNPV *lef-5* knockout and repair bacmids. A: Construction of an *Ac*MNPV *lef-5* knockout bacmid (bAc^{lef5ko}). The top diagram shows the Wt AcMNPV *lef-5* locus and illustrates the orientations and overlap among *38K*, *lef-5*, and *p6.9* ORFs. To generate the *lef-5* knockout bacmid (bAc^{lef5ko}), a PCR fragment (lef5ko) was amplified and used for long-primer PCR and lambda RED recombinase mediated replacement of the indicated portion of the *lef-5* ORF. The central portion of the *lef-5* ORF was replaced with a cassette containing a late promoter (*p6.9*)-driven *GUS* gene, and a chloramphenicol resistance (*cat*) cassette, as illustrated. Hatched boxes represent *lef-5* ORF sequences and numbers in bold indicate AcMNPV genome positions. B: Construction of *lef-5* repair bacmids,*lef-5* repair and control bacmids were constructed by insertion of a cassette into the polyhedrin (PH) locus of the *lef-5* knockout bacmid (bAc^{lef5ko}) shown in panel A. Using pFastBac vectors (names are indicated in parenthesis beside each diagram), cassettes encoding and expressing the following *lef-5* gene or control constructs were used to generate bacmids: cMyc-tagged AcMNPV LEF-5 (bAc$^{lef5ko/FB\text{-}mycAclef5}$), EGFP-tagged AcMNPV LEF-5 (bAc$^{lef5ko/FB\text{-}EGFPAclef5}$) and EGFP-tagged SeMNPV LEF-5 (bAc$^{lef5ko/FB\text{-}Selef5EGFP}$), or an empty cassette with no *lef-5* ORF (bAc$^{lef5ko/FB}$). Both cMyc- and EGFP-tagged *lef-5* fusion genes are under the control of the OpMNPV *ie1* promoter (*Opie1P*).

To rescue the *lef-5* knockout bacmid (bAc^{lef5ko}) and to confirm that effects of the knockout resulted from the absence of the *lef-5* gene, we generated three repaired bacmids by inserting various forms of the *lef-5* gene into the polyhedrin locus of bAc^{lef5ko} (Fig. 1B). Two forms of the AcMNPV *lef-5* gene were used to repair bAc^{lef5ko} : an AcMNPV *lef-5* gene encoding an N-terminal c-myc epitope tag (*mycAclef5*), and an

AcMNPV *lef-5* gene encoding an N-terminal EGFP fusion (*egfpAclef5*). In addition, we inserted a SeMNPV *lef-5* construct that was EGFP tagged at the C-terminus (*Selef5egfp*). SeMNPV was previously shown to complement AcMNPV late transcription in a transient late transcription assay (Berretta and Passarelli, 2006). Therefore, we asked whether SeMNPV *lef-5* would complement the AcMNPV *lef-5* knockout virus. In each of the three repair bacmids, the *lef-5* construct was expressed under the control of an OpMNPV *ie-1* promoter (Fig. 1B, *Opie1P*). pFastBac plasmids constructed for insertion of *lef-5* constructs into the polyhedrin locus are shown in Fig. 1B (pFB-mycAclef5, pFB-EGFPAclef5 and pFB-Selef5EGFP) and the resulting bacmids that express MycAcLEF5, EGFPAcLEF5, and SeLEF5EGFP, were designated as bAc$^{lef5ko/FB\text{-}mycAclef5}$, bAc$^{lef5ko/FB\text{-}EGFPAclef5}$, and bAc$^{lef5ko/FB\text{-}Selef5EGFP}$, respectively. A late promoter-driven reporter gene (*p6.9-GUS*) was also included in each pFastBac plasmid described above. A pFastBac plasmid containing a reporter gene but no *lef-5* gene was also used to generate an "empty vector" control bacmid, designated as bAc$^{lef5ko/FB}$ (Fig. 1B). Expression of each of the *lef-5* fusion constructs (MycAcLEF5, EGFPAcLEF5, and SeLEF5EGFP) was confirmed by Western blot analysis (Fig. 2B–E).

To examine the role of LEF-5 in the context of an AcMNPV infection, Sf9 cells were transfected with the *lef-5* knockout bacmid (bAc^{lef5ko}) or a repaired bacmid (bAc$^{lef5ko/FB\text{-}mycAclef5}$). No GUS activity was detected from the late promoter-driven GUS (*p6.9-GUS*) reporter in Sf9 cells at 72 h post transfection (p.t.) with the bAc^{lef5ko}. However, in cells transfected with the *lef5*-repair bacmid bAc$^{lef5ko/FB\text{-}mycAclef5}$, late promoter driven GUS expression was readily observed (Fig. 3). Transfection–infection experiments were next conducted by using the supernatants from Sf9 cells transfected with bAc^{lef5ko} or bAc$^{lef5ko/FB\text{-}mycAclef5}$ to infect Sf9 cells. Late promoter driven GUS expression was observed in cells infected with the repaired virus (vAc$^{lef5ko/FB\text{-}mycAclef5}$) at 48 h p.i., but not in cells exposed to supernatants from cells transfected with the *lef-5* knockout bacmid (bAc^{lef5ko}) (Fig. 3, lower panels). An additional control bacmid (bAc$^{lef5ko/FB}$) that contains a *lef-5* knockout plus an empty cassette in the polyhedrin locus, was also negative for *P6.9*-driven GUS expression (data not shown). Using the same assay, the *lef-5* knockout virus was also rescued by the two other *lef-5* constructs: EGFP-tagged AcMNPV LEF-5 and EGFP-tagged SeMNPV LEF-5 (bacmids bAc$^{lef5ko/FB\text{-}EGFPAclef5}$ and bAc$^{lef5ko/FB\text{-}Selef5EGFP}$, respectively) (data not shown). For these studies, successful transfection of bAc^{lef5ko} in Sf9 cells was confirmed by immunofluorescence detection of the GP64 protein (data not shown), which is normally expressed from both early and late promoters. Thus, the *lef-5* knockout bacmid was defective for late reporter gene expression and viral replication, but was rescued by cMyc- and EGFP-tagged AcMNPV LEF-5 proteins, and by EGFP-tagged SeMNPV LEF-5 protein. These results indicate that the *lef-5* gene is necessary for AcMNPV late gene expression and viral replication, and that a SeMNPV *lef-5* gene rescued both functions in the *lef-5* knockout virus.

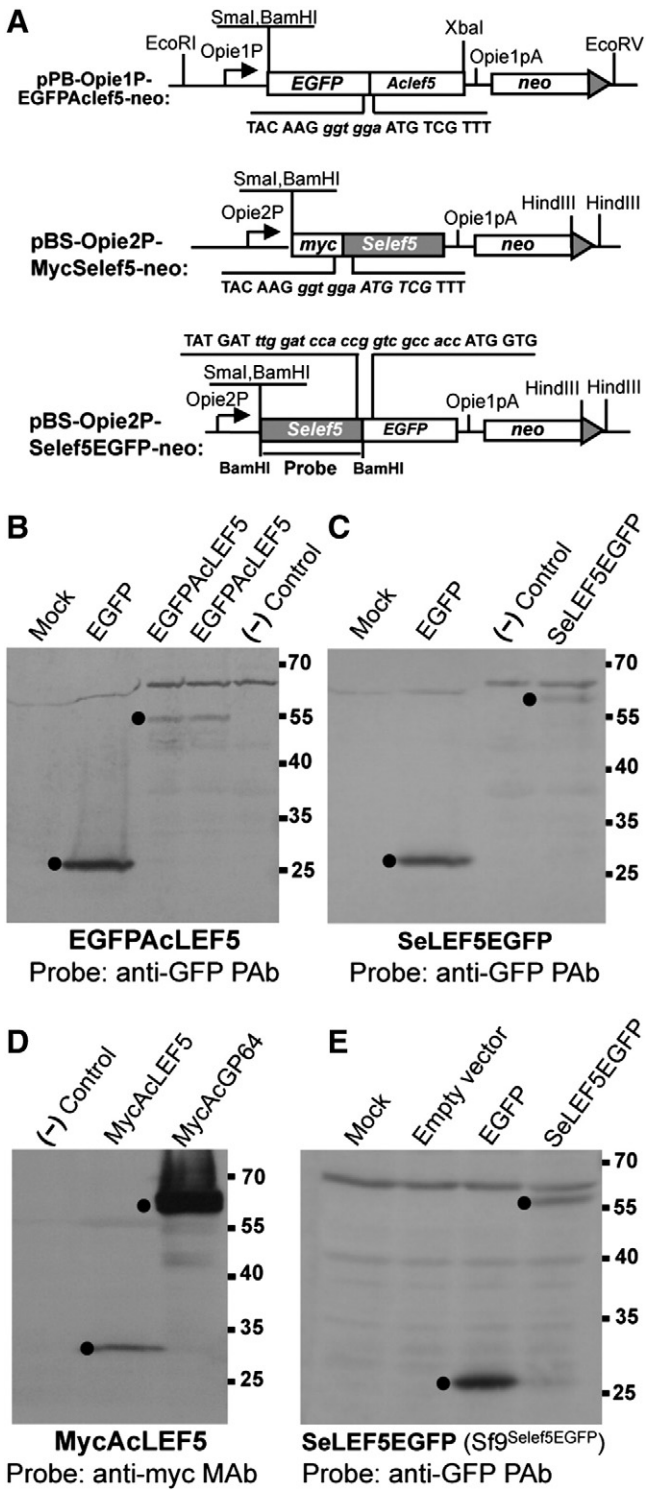

Fig. 2. Western blot analysis of LEF-5 fusion proteins: EGFPAcLEF5, SeLEF5EGFP, and MycAcLEF5. Construction of genes encoding cMyc- and EGFP-tagged LEF-5 proteins are shown in panel A. Sequences at the junction of the fusion are indicated above or below the diagram and nucleotides in lower case italic represent codons encoding additional amino acids between the two ORFs. A neomycin resistance (*neo)* gene is expressed under the control of an OpMNPV *gp64* promoter and flanked by an SV*40* poly(A) site. (Abbreviations: *Opie1P*, OpMNPV *ie-1* promoter; Opie1-pA, OpMNPV *ie-1* poly A site; *Opie2P*, truncated OpMNPV *ie-2* promoter). The BamHI fragment of plasmid pBS-Opie2P-Selef5EGFP-neo was used as a probe for Southern blot analysis of the SeLEF5EGFP-expressing cell lines (Probe). An anti-GFP polyclonal antibody was used to detect LEF5-EGFP fusion proteins from the nuclear fractions of Sf9 cells infected with virus vAc$^{lef5ko/FB\text{-}EGFPAclef5}$ expressing EGFP-tagged AcMNPV LEF-5 protein (Panel B; EGFPAcLEF5) or virus vAc$^{lef5ko/FB\text{-}Selef5EGFP}$ expressing EGFP-tagged SeMNPV LEF-5 protein (Panel C, SeLEF5EGFP). As a positive control, EGFP expressed in Sf9 cells transfected with plasmid pPB-Opie1P-EGFP-neo was detected from cell lysates (Panels B, C, E; EGFP). An anti-cMyc monoclonal antibody was used to detect cMyc-tagged AcMNPV LEF-5 from the cell lysates of Sf9 cells infected with virus vAc$^{lef5ko/FB\text{-}MycAclef5}$ (Panel D, MycAcLEF5). As a positive control, MycAcGP64 was collected from the cell lysates of Sf9 cells infected with virus vAc^{MycAcGP64}. Proteins extracted from the nuclear fraction (Panels B,C) or cell lysates (Panel D) of vAc$^{lef5ko/FB}$ and Wt AcMNPV coinfected Sf9 cells were used as a negative (−) control. Proteins collected from an empty vector pPB-Opie1P-neo transfected Sf9 cells were used as a negative control (Panel E) for detection of SeLEF5EGFP fusion protein in the cell line Sf9^{Selef5EGFP}. EGFP- or Myc-specific bands are indicated by black dots and correspond to the predicted masses of EGFP (27 kDa), EGFP-AcLEF5 (58 kDa), SeLEF5EGFP (60 kDa), and MycAcLEF5 (32 kDa).

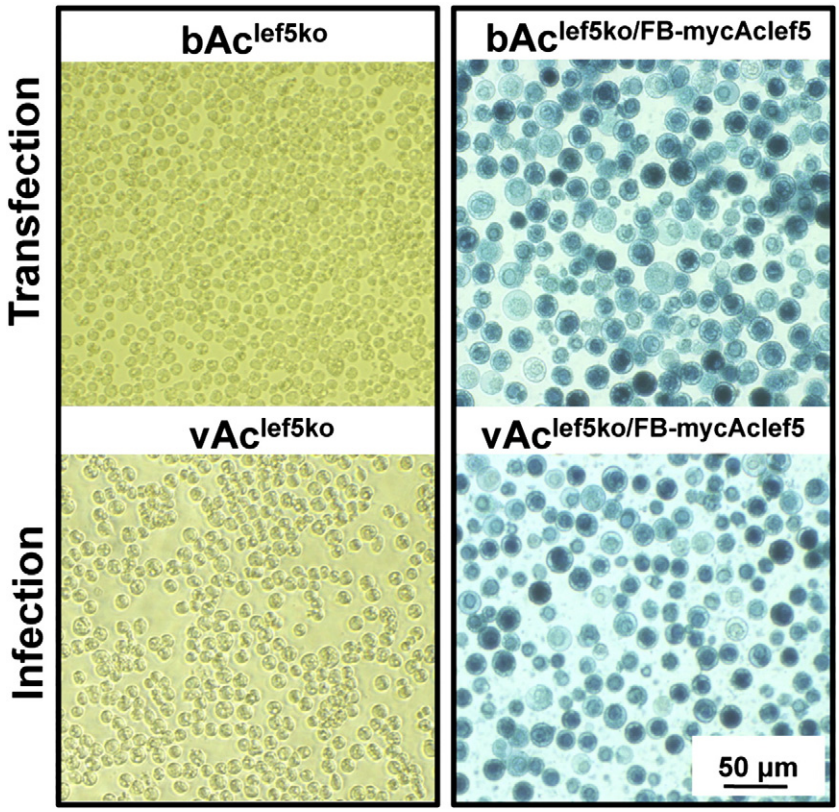

Fig. 3. Transfection–Infection analysis of *lef-5* knockout and repair bacmids: Rescue of late gene expression and viral replication by cMyc-tagged AcMNPV Lef-5. Sf9 cells were transfected with either the *lef-5* knockout bacmid (bAc^{lef5ko}) or a "repair" bacmid that expresses a cMyc-tagged AcMNPV LEF-5 protein (bAc$^{lef5ko/FB\text{-}mycAclef5}$). *P6.9* promoter-driven GUS expression was detected at 72 h p.t. (upper panels) by incubation with X-Gluc substrate. Supernatants were removed from transfected cells (upper panels) at 72 h p.t. and transferred to Sf9 cells, which were subsequently incubated for 48 h and then stained by incubation with X-Gluc to identify infected cells (lower panels). An additional control *lef-5* knockout bacmid (bAc$^{lef5ko/FB}$), derived from bAc^{lef5ko} but containing an empty cassette in the polyhedrin locus, was also examined. Similar to the results from bAc^{lef5ko}, we detected no *P6.9*-driven GUS expression from bAc$^{lef5ko/FB}$ (data not shown).

A stable cell line expressing a SeMNPV LEF-5 EGFP fusion rescues the lef-5 knockout virus

Because the *lef-5* gene is necessary for AcMNPV replication, we generated a LEF-5 expressing cell line for propagating the *lef-5* knockout virus (vAc^{lef5ko}). Since the vAc^{lef5ko} retains coding sequences from the 5′- and 3′- ends of the *Ac*MNPV *lef-5* gene, we used the heterologous SeMNPV *lef-5* gene (*Selef-5*) for generation of stably-transfected cells expressing LEF-5. The heterologous SeMNPV *lef-5* gene was used in order to avoid rapidly generating revertant viruses through homologous recombination. Sequence identity between the *Selef-5* and *Aclef-5* gene is 58%. We generated cell lines by transfecting Sf9 cells with a plasmid (pBS-Opie2P-Selef5EGFP-neo, Fig. 2A) that contains the SeMNPV *lef5-egfp* fusion gene under the control of a truncated OpMNPV *ie2* promoter (Theilmann and Stewart, 1992) and selecting cells in G418, as described previously (Lin and Blissard, 2002b). Cells expressing an integrated SeMNPV *lef5-egfp* gene were identified by EGFP fluorescence (Fig. 4A) and cloned as single cells. A cell line (P4A10-sub8) expressing the SeMNPV LEF5-EGFP fusion (SeLEF5EGFP) was designated as Sf9^{Selef5EGFP}. Expression of the SeLEF5EGFP fusion protein was confirmed by Western blot analysis using an anti-GFP polyclonal antibody (Fig. 2E). In addition, by cotransfecting Sf9 cells with plasmids expressing the two proteins, we found that the SeLEF5EGFP protein colocalized with a cMyc-tagged SeMNPV LEF-5 protein (MycSeLEF5) in the nuclei of cells (Fig. 4B) as expected. This suggests that fusion of EGFP to SeMNPV LEF-5 did not interfere with its nuclear localization, and that the SeLEF5EGFP protein should be appropriately localized in the cell line, Sf9^{Selef5EGFP}.

To determine if SeLEF5EGFP expression in cell line Sf9^{Selef5EGFP} was sufficient to rescue late transcription, those cells were transfected with bacmid bAc^{lef5ko}. GUS activity was detected in transfected Sf9^{Selef5EGFP} cells (Fig. 4C). In contrast, no GUS activity was detected in control Sf9 cells transfected with the same bAc^{lef5ko} (Fig. 4C, Sf9). Thus, late transcription was rescued by the SeMNPV LEF-5 construct. To determine if the stably transfected cells supported viral replication by the *lef-5* knockout virus, and whether virions produced in that cell line were infectious, supernatants were collected from Sf9^{Selef5EGFP} cells that were transfected with the knockout bacmid (above), and were subsequently used to infect both Sf9 and Sf9^{Selef5EGFP} cells. At 3 days p.i. P6.9-driven GUS expression was detected in the SeLEF5EGFP-expressing cell line (Sf9^{Selef5EGFP}) but not in Sf9 cells (Fig. 4C). These results indicate that infectious virus was generated in the SeLEF5EGFP-expressing cell line. Titers of vAc^{lef5ko} generated in cell line Sf9^{Selef5EGFP} ranged from 1.6×10^6 I.U./ml to 1.6×10^7 I.U./ml after two rounds of amplification. We also examined the vAc^{lef5ko} virus prepared in the SeLEF5EGFP-expressing cell line for revertant viruses. A high titer viral stock (1.6×10^7 I.U./ml) was used to infect Sf9 cells at an MOI of 5 and cells were assayed for GUS activity at 5 days p.i. Approximately 6 GUS-positive cells per million cells were detected in the infected Sf9 cells. In contrast, most cells were GUS-positive when the SeLEF5EGFP-expressing cell line was infected in parallel, as a positive control (data not shown). These results indicate that the SeLEF5EGFP-expressing cell line can complement late gene expression in the vAc^{lef5ko} virus and can be used to generate high titer vAc^{lef5ko} viruses. While some revertants appear to be generated, the low level of detection (one revertant per app. 2.5×10^5 particles)

appears to be acceptable for analysis of the *lef-5* knockout in one-step experiments.

Viral replication is defective in the lef-5 knockout

To characterize replication of vAc^{lef5ko} in the SeMNPV LEF-5 (SeLEF5EGFP) expressing cells, the vAc^{lef5ko} virus (generated in the SeLEF5EGFP-expressing cell line Sf9^{Selef5EGFP}) was used to infect Sf9^{Selef5EGFP} cells and control Sf9 cells at an MOI of 5. A one-step growth curve was then generated to monitor infectious virus production. As an additional control, the repair virus vAc$^{lef5ko/FB\text{-}mycAclef5}$ was also used to infect both SeLEF5EGFP-expressing cells and Sf9 cells. A control virus (vAc$^{Wt/FB\text{-}mycEGFP}$) containing a Wt *lef-5* locus was also

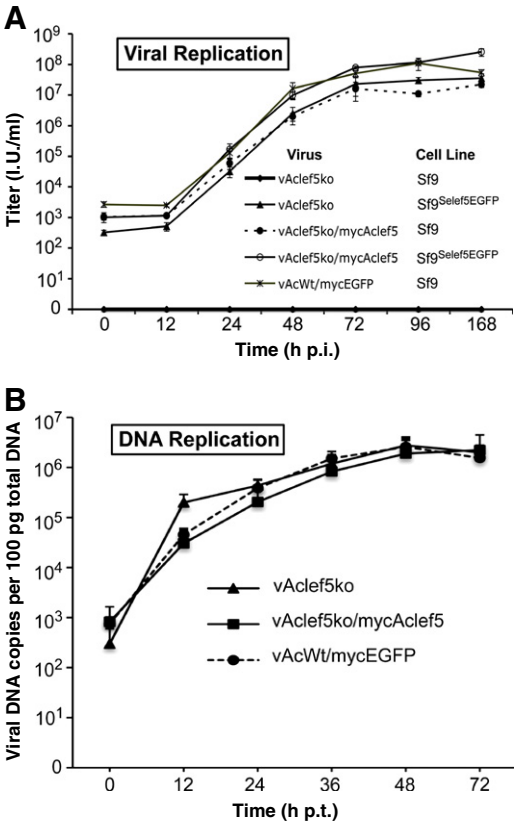

Fig. 5. Analysis of viral replication and viral DNA replication. A. Viral replication was examined by generating viral one-step growth curves in either Sf9 cells or in SeLEF5EGFP-expressing cells (Sf9^{Selef5EGFP}). The *lef-5* knockout (vAc^{lef5ko}) or repair (vAc$^{lef5ko/FB\text{-}mycAclef5}$) virus was used to infect each cell line (MOI of 5) in triplicate. A control virus (vAc$^{Wt/FB\text{-}mycEGFP}$) with a Wt *lef-5* locus but harboring a *myc-egfp* cassette in the polyhedrin locus, was also used to infect Sf9 cells in parallel. Error bars represent standard deviation from the mean. (I.U., infectious units). B. Viral DNA replication. To measure AcMNPV DNA replication in the absence of LEF-5, DNA levels were measured in Sf9 cells infected with either a *lef-5* knockout virus (vAc^{lef5ko}) or two control viruses (vAc$^{lef5ko/FB\text{-}mycAclef5}$, and vAc$^{Wt/FB\text{-}mycEGFP}$) that express LEF-5. Sf9 cells were infected at an MOI of 5 in triplicate with virus vAc^{lef5ko}, vAc$^{lef5ko/FB\text{-}mycAclef5}$, or vAc$^{Wt/FB\text{-}mycEGFP}$. Viral DNA accumulation in each infection was assayed at various times post infection, by qPCR with an *odv-e56* primer set (Table 1) as described in the Materials and Methods. Each value represents the average of triplicate infections, and error bars represent standard deviation from the mean. Virus abbreviations: vAclef5ko = vAc^{lef5ko}; vAclef5ko/mycAclef5 = vAc$^{lef5ko/FB\text{-}mycAclef5}$; vAcWt/mycEGFP = vAc$^{Wt/FB\text{-}mycEGFP}$.

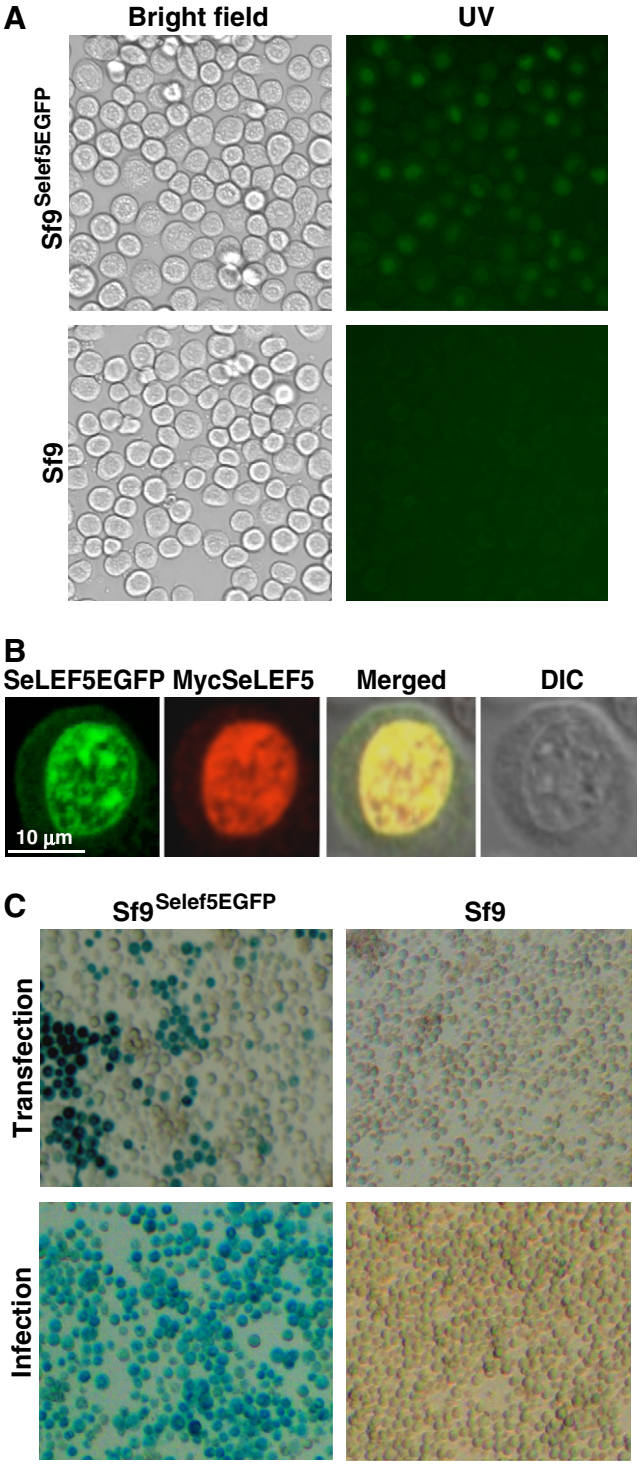

Fig. 4. Rescue of an AcMNPV *lef-5* knockout by a stable cell line expressing a SeMNPV LEF5-EGFP fusion protein. A. Expression of the SeMNPV LEF5-EGFP fusion protein (SeLEF5EGFP) was detected in SeLEF5EGFP-expressing cell line (Sf9^{Selef5EGFP}) by epifluorescence microscopy. The majority of the SeLEF5EGFP-expressing cells (Sf9^{Selef5EGFP}) showed fluorescence that was localized to the nucleus (upper right panel), while control Sf9 cells show no apparent fluorescence (lower right panel). Bright field (left) and UV fluorescence (right) images are shown. B. Expression and cellular localization of EGFP-tagged SeMNPV LEF-5 (SeLEF5EGFP) and cMyc-tagged SeMNPV LEF-5 (MycSeLEF5) were examined at 48 h p.t. in Sf9 cells co-transfected with plasmids pBS-Opie2P-Selef5EGFP-neo and pBS-Opie2P-MycSelef5-neo. MycSeLEF5 was detected by immunofluorescence using an anti-cMyc MAb and an Alexa Fluor 594 goat anti-mouse IgG (Invitrogen). C. Rescue of late gene expression and infectivity by stably transfected Sf9 cells expressing a SeMNPV LEF5-EGFP fusion protein. DNA from the *lef-5* knockout bacmid (bAc^{lef5ko}) was used to transfect either cell line Sf9^{Selef5EGFP} or Sf9 cells, and GUS expression was detected by addition of X-Gluc substrate at 5 days p.t.. Prior to GUS detection, supernatants from each well of transfected cells were transferred to cells of the same type (Sf9^{Selef5EGFP} or Sf9 cells, respectively) then incubated for 3 days and again assayed for GUS activity. GUS expression from the *lef-5* knockout virus was detected only in the Sf9^{Selef5EGFP} cells.

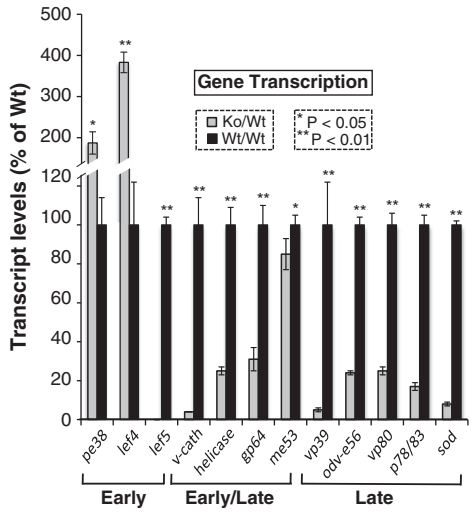

Fig. 6. Effects of the *lef-5* knockout on transcripts of representative early, early/late, and late genes. The effect of disrupting *lef-5* on transcription of several representative early, early + late, and late genes was measured by RT-qPCR of viral transcripts at 48 h p.i. The transcript level of each gene was normalized to the viral genome copy number and the effects of the *lef-5* knockout are shown for each gene as the percentage of detected transcripts relative to that of the Wt control virus (vAc$^{Wt/FB\text{-}mycEGFP}$). Data points and error bars represent means and standard deviations from triplicate infection data. The early/late gene, *pe38*, is indicated as an early gene here because the primer set selected for this assay detects only the early transcript. (Virus abbreviations: Ko = vAc^{lef5ko}; Wt = vAc$^{Wt/FB\text{-}mycEGFP}$).

included in this study. Detection of the GUS reporter was used to assess viral infection. No virus replication was detected in the vAc^{lef5ko}-infected Sf9 cells (Fig. 5A), indicating that LEF-5 is essential for AcMNPV viral replication. However, in the SeLEF5EGFP-expressing cell line, the vAc^{lef5ko} virus replicated in a manner similar to that of the control viruses in Sf9 cells or in the SeLEF5EGFP-expressing cell line.

Viral DNA replication is unaffected by the lef-5 knockout

In prior studies, the role of *lef-5* was examined in late transcription assays of *lef* genes transiently expressed from plasmids, or by *in vitro* assays using purified proteins. To determine if LEF-5 might also play a role in viral DNA replication in the context of a viral infection, we infected Sf9 cells with the *lef-5* knockout virus (vAc^{lef5ko}) and monitored viral DNA replication in those cells using qPCR. Viral DNA levels were monitored at various times (0, 12, 24, 36, 48, 72 h) post infection, and these data were compared with similar data from control viruses consisting of a repair virus (vAc$^{lef5ko/FB\text{-}mycAclef5}$) and a virus with a Wt *lef-5* locus (vAc$^{Wt/FB\text{-}mycEGFP}$). Viral DNA replication of the *lef-5* knockout virus (vAc^{lef5ko}) was similar to that from the two control viruses that express LEF-5 (Fig. 5B). Thus, when the *lef-5* gene was disrupted, we observed no substantial effect on viral DNA replication in virus (vAc^{lef5ko})-infected Sf9 cells over the normal timecourse of the infection cycle.

Effects of a lef-5 knockout on AcMNPV transcription

The AcMNPV *lef-5* gene is important for baculovirus late promoter–reporter expression in a transient expression system (Passarelli and Miller, 1993c), and LEF-5 stimulates late transcription by *in vitro* transcription assays (Guarino et al., 2002a). To examine the effect of disruption of the *AcMNPV lef-5* gene on late gene expression in the context of an AcMNPV infection, we infected Sf9 cells with *lef-5* knockout virus vAc^{lef5ko} and analyzed specific AcMNPV transcripts at various times post infection by reverse transcription quantitative PCR (RT-qPCR). We examined genes that represent regulation by early (*lef-4*, and *pe38*), early + late (*v-cath*, *helicase*, *gp64*, *me53*), and late (*vp39*, *odv-e56*, *vp80*, *p78/83*, *sod*) promoters. The effects of the *lef-5* knockout on transcripts from these genes are shown as percent changes relative to the transcript levels detected from Sf9 cells infected with a control virus that contains a Wt *lef-5* gene (virus vAc$^{Wt/FB\text{-}mycEGFP}$) (Fig. 6). The measured transcript numbers (copies per thousand viral genomes) are listed in Table S-1 (Supplementary Data). When *lef-5* was absent, transcript levels of late genes (*vp39*, *odv-e56*, *vp80*, *p78/83*, *and sod*) were reduced by 75% to 95%. Similarly, transcripts from genes classified as early/late (*v-cath*, *helicase*, *gp64*, *me53*) were reduced by 15% to 96%. Unexpectedly, disruption of the *lef-5* gene resulted in approximately 2–4 fold increases in the *pe38* and *lef-4* early transcripts, respectively. (Note: Although *pe38* is also classified as an early/late gene (Krappa et al., 1995; Mans and Knebel-Moersdorf, 1998; Wu et al., 1993), the primer set selected for this analysis detects only the early transcript). As expected, the transcript of knockout gene *lef-5* was undetectable in the vAc^{lef5ko} virus-infected Sf9 cells since the primer set for *lef-5* was within the region deleted from the coding sequence (Table 1, Fig. 1). Thus, these data show that in the absence of LEF-5, late transcripts are dramatically reduced, indicative of a direct role of LEF-5 in the production or stability of late transcripts.

LEF-5 colocalizes with IE1 in the nucleus

While viral replication, and late transcription occur within the nuclei of infected cells, the nuclear subdomains associated with various functions remain poorly defined. To begin the examination of LEF-5 in the context of such associations, we examined the nuclear localization of LEF-5 in relation to *IE-1*, a transcription factor necessary for early gene expression and DNA replication. As described above, we found that EGFP-tagged LEF-5 constructs rescued infectivity of the *lef-5* knockout virus, and resulted in moderate to high titer viruses of a repair virus (vAc$^{lef5ko/FB\text{-}EGFPAclef5}$). In addition, we also found that EGFP- and cMyc-tagged LEF-5 proteins colocalized (Figs. 4B and 7A). To examine localization of the LEF-5 protein (EGFPAcLEF5) in relation

Table 1
Primers used for analysis of gene transcription.

Names of genes	Phase of transcription	Forward primers	Reverse primers	cDNA primers
pe38	E	CAAATGCGTCATCAATCTGC	CACGTCACAACAGCGTTAGG	CAGCTTCCAAAGCAGCTATCTTTGCC
lef4	E	TCGAAAATTTGAACGGGAAC	GCAAAAATTGCGCATAAACA	CGATGGCCGTCACCGGATCTATAG
lef5	E	TGGTGAAAGAGCGCAAACAAATTCG	GCAATAGCTGGCACGGACAACAG	TGCGTGTACACCCAGTGCTG
v-cath	E/L	CTGGCGTCGTCTCAACAAAGTCACTAG	AGCCAGCGTCGACAAAATCACAATC	CAATGTCGGCAGCGTCTATGG
helicase	E/L	ACCACGTTGCCGCAGACATTC	TGGCGATGTGATAAGTTCTATAAATGC	GCTGTTCGTGCGAATCAACG
gp64	E/L	CCACCAAAGGCGACCTGATGC	TCAGGTCGTGCAGCATATTGTTTAGC	CAATTGCTAGTTCCTTGTAGTTGC
me53	E/L	AAATCACAAAGAGCCCAACG	ACAGTTGACGTCATCCACCA	GCGCCGTCCAATACCTGGGT
vp39	L	CAACGAAAACGCAGTTAACACTATATGC	CGTGTTCGGGTTTGTGGTGTCC	CTAAACCTCAATTCCTCCGTGTC
odv-e56	L	GATCTTCCTGCGGGCCAAACACT	AACAAGACCGCGCCTATCAACAAA	TTGCATAGAAATAACAATGGGAG
vp80	L	CGAACATTACACCGATCAGGACAAAG	TGATCAATAGTTGTTATGTGCAACCGA	GCTCGAATCTTTAATTTCTTGCG
p78/83	L	CCTCCACCACCACCACCACCA	GCGATTTTTTTCGTTTCTAATAGCTTCC	CCTCGTCGTTAGAAGTTGCTTC
sod	L	TGAAAGCCATCTGCATCATTAGCG	TTGCTCGTGTCGCCATATTCGTG	TTGCTCAACGGATGATCGGTAAG

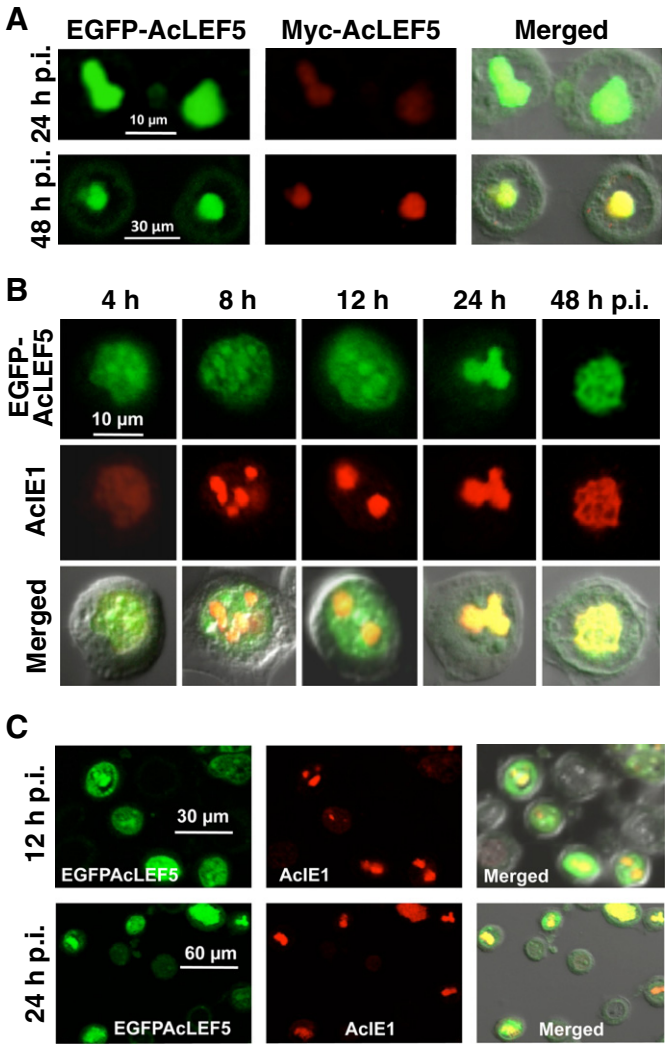

Fig. 7. Subcellular localization of EGFP-tagged AcMNPV LEF-5 and IE1 in infected Sf9 cells. A. Colocalization of EGFP-LEF5 (EGFPAcLEF5) and cMyc-LEF5 (Myc-AcLEF5). Sf9 cells were infected with viruses expressing EGFP- and cMyc-tagged LEF-5 (vAc$^{lef5ko/FB\text{-}EGFPAclef5}$ and vAc$^{lef5ko/FB\text{-}MycAclef5}$, respectively). cMyc-tagged LEF-5 was detected by immunofluorescence microscopy using an anti-cMyc MAb and compared with EGFP fluorescence from the EGFPAcLEF5 protein. B. Subcellular localization of LEF-5 and IE-1 in AcMNPV infected cells at various times post infection. Sf9 cells were infected with virus vAc$^{lef5ko/FB\text{-}EGFPAclef5}$, which expresses an EGFP-tagged LEF-5 protein. The EGFPAcLEF5 protein was detected by EGFP fluorescence and IE1 was detected by immunofluorescence microscopy using an anti-IE1 MAb. A representative cell is shown at each of the indicated time points post infection. C. Localization of EGFP-LEF-5 and IE-1 in AcMNPV infected cells is shown in multiple cells at lower magnification, at 12 and 24 h p.i.

to the IE-1 protein (AcIE1), Sf9 cells were infected with virus vAc$^{lef5ko/FB\text{-}EGFPAclef5}$, which expresses the EGFP-tagged AcMNPV LEF-5 protein and WT IE-1 (AcIE1). AcIE1 was detected by immunofluorescence microscopy with an anti-IE-1 MAb (Knebel-Moersdorf et al., 2006). Both EGFPAcLEF5 and AcIE1 were detected as early as 4 h p.i., as diffuse fluorescence throughout the infected cell nucleus, with some areas of apparently moderate concentration (Fig. 7B). By 8 and 12 h p.i., IE-1 staining was found primarily in discrete nuclear structures and EGFPAcLEF5 showed some colocalization but generally appeared more diffuse. By 24 h p.i. the AcIE-1 protein was found coalesced into large structures (Fig. 7B). At this time EGFPAcLEF5 was similarly concentrated, less diffuse, and colocalized with AcIE1. The dramatic change in the colocalization pattern between 12 and 24 h p.i. is illustrated in a lower magnification view in Fig. 7C. By 48 h p.i., EGFPAcLEF5 and AcIE1 remained colocalized but were often found within a large net-like structure within a subdomain of the now expanded cell nucleus (Fig. 7B, 48 h p.i.). Thus, co-expression of EGFPAcLEF5 and AcIE1 in the context of an infected cell resulted in close co-localization of these two proteins in the nucleus of infected cells.

Discussion

In the current study, we used a bacmid-based AcMNPV knockout system to examine the role of LEF-5 in the context of the AcMNPV infection cycle. To evaluate the requirement for LEF-5, we inactivated the *lef-5* gene in the AcMNPV genome by removing a substantial portion of the *lef-5* ORF in bacmid bMON14272. Because the *lef-5* ORF overlaps the adjacent *38K* and *p6.9* genes, we avoided disrupting those flanking genes by retaining N- and C-terminal portions of the *lef-5* ORF in the knockout virus. We also generated a complementing stable cell line in order to propagate the *lef-5* knockout virus. Because, inclusion of the AcMNPV *lef-5* gene in the genome of stably transfected Sf9 cells would likely lead to homologous recombination and rapid spontaneous rescue of the *lef-5* knockout virus, we generated a stable cell line that expresses the SeMNPV *lef-5* gene as a LEF-5-EGFP fusion. The nt sequence of SeMNPV *lef-5* gene differs substantially from that of AcMNPV *lef-5* and the SeMNPV *lef-5* gene was previously shown to substitute for AcMNPV *lef-5* in a transient late transcription assay (Berretta and Passarelli, 2006). While we did detect a low level of spontaneously rescued virus in stocks prepared in the SeMNPV *lef-5* expressing cell line, we found that revertant virus was present at a very low level, less than one in app. 2.5×10^5 particles.

Using the AcMNPV bacmid containing the *lef-5* knockout to initiate infection in Sf9 cells by transfection, we found that the *lef-5* knockout bacmid was unable to support late gene expression from a late *p6.9* promoter–reporter construct, and was also unable to initiate productive infection. However, when AcMNPV or SeMNPV *lef-5* constructs were inserted into the polyhedrin locus of the same *lef-5* knockout bacmid, the bacmids and the resulting repair viruses were viable and initiated a robust infection in Sf9 cells. This demonstrates that the defect in viral replication was due to the loss of *lef-5* expression and not the indirect effects of the knockout construction. Further examination showed that while the *lef-5* knockout prevented viral replication, it did not have any substantial negative effect on the level of viral DNA replication. In contrast however, using RT-qPCR we found that the *lef-5* knockout had a dramatic negative effect on the level of transcripts from representative late genes, and from genes transcribed by early + late promoters. Interestingly, transcript levels from two representative early genes were higher than that in a control virus expressing WT LEF-5. The observed increase in early gene transcripts in the absence of *lef-5* may have resulted from absence of normal down-regulation of some early transcription during the late phase. Consistent with that model, an earlier report found that *lef-4* mRNA levels peaked around 9 h p.i. and decreased afterwards (Durantel et al., 1998). Also, in a prior study of the AcMNPV DNA binding protein (DBP), knockdown of DBP using RNAi also resulted in substantial (2–5 fold) increase in transcripts of several early genes including *lef-3*, *lef-4*, and *P35* (Quadt et al., 2007). In the absence of LEF-5, we observed a severe reduction, but not a complete absence of late transcripts from several late genes (*p78/83*, *vp80*, and *odv-e56*). The detection of low levels of these late transcripts by RT-qPCR could result from either a) very low levels of late transcription from the late promoters, or b) transcripts initiated from early promoters located upstream or downstream of the late gene. Prior studies using *in vitro* transcription assays suggested that LEF-5 serves as a late transcription initiation factor (Guarino et al., 2002a). While our data indicate that LEF-5 is required for productive viral infection and has a dramatic effect on late transcript levels, it is not yet clear whether in some cases, late transcription may be initiating at a low frequency in the

absence of LEF-5. It will be of interest to examine these questions in future studies.

LEF-5 constructs co-localized with AcMNPV IE-1 in the nuclei of infected cells. Because LEF-5 is likely to be associated with the late RNA polymerase complex at or near late promoters on viral DNA, and IE-1 binds to *hr* elements (enhancers of early transcription and origins of replication) on the viral genome, it is perhaps not surprising that both colocalize in the nuclei of infected cells. While the pattern of nuclear localization was generally similar throughout infection, some differences were observed. For example, at 8 and 12 h p.i. the EGFPAcLEF5 construct appears to be found in a more diffuse nuclear pattern than that of AcIE1, whereas before (4 h p.i.) and after (24 and 48 h p.i.) this period the two proteins appear very highly similar in localization. Several factors may explain these differences: First, the EGFP-AcLEF5 construct was expressed from an OpMNPV *ie1* promoter. While this is an early promoter, the timing and level of LEF-5 expression may differ somewhat from that of WT AcMNPV LEF-5. Second, the EGFP used to tag LEF-5 may be responsible for some minor differences in localization, although we did not detect any substantial differences in localization between the EGFP-tagged LEF-5 and a LEF-5 tagged with a small epitope (cMyc). Finally, it is also possible that the differences in sensitivity of detection (EGFP vs. immunofluorescence detection) may result in some differences in apparent localization during the 12–24 h p.i. period. Because prior studies (Guarino et al., 2002a; Harwood et al., 1998) suggest that LEF-5 is likely to be directly associated with the late RNA polymerase complex and/or with viral late promoters, it is also possible that the observed differences may reflect functional differences between the known roles of IE-1 (as transcription factor, enhancer binding protein, and origin binding protein) and that of LEF-5 in regulating or modulating late promoter or RNA polymerase activity.

In the current study, we describe the generation and application of an experimental system for examining the function of LEF-5 in the context of the AcMNPV infection. We have demonstrated that in this context, LEF-5: a) is necessary for viral replication, b) has no apparent effect on viral DNA replication, c) appears to co-localize closely with IE-1 in the nucleus during most of the infection cycle, and d) has a dramatic effect on viral late transcripts, consistent with prior studies of its likely very direct role in late transcription. This system should permit future studies to examine in more detail, the precise role(s) and mechanistic function of LEF-5 in facilitating or modulating AcMNPV late transcription.

Materials and methods

Plasmid construction

To generate LEF-5 expressing cell lines and to analyze subcellular localization of AcMNPV and SeMNPV LEF-5 proteins, we constructed several plasmids containing either *egfp*- or epitope (*cMyc*)-tagged *lef-5* fusions (*egfp-Aclef5* and *myc-Aclef5; Selef5-egfp* and *myc-Selef5*). Plasmids were assembled by PCR amplification and subcloning. Primers used for PCR and plasmid construction are listed in Table S-2 (Supplementary Data). To generate LEF-5 expressing cell lines, we assembled a plasmid construct containing the *Opie2* promoter driving expression of an *egfp-lef5* fusion, using the SeMNPV *lef-5* ORF (*Selef5-egfp*). The plasmid also contains a neomycin phosphotransferase gene (*neo*) under the control of an OpMNPV *gp64* early promoter (*OpGP64*). The resulting plasmid pBS-Opie2P-Selef5EGFP-neo (Fig. 2A) contains a *lef-5* cassette with 280 bp (from −259 to +21) from the OpMNPV *ie2* promoter (Theilmann and Stewart, 1992) and 1578 bp from the *egfp* ORF fused to the SeMNPV *lef-5* ORF at amino acid 1. The antibiotic resistance cassette consists of 166 bp (from −166 to −1) from the OpMNPV *gp64* promoter (Blissard and Rohrmann, 1991) and the 795 bp *neo* ORF (Monsma et al., 1996). To generate a plasmid for analysis of the subcellular localization of cMyc-tagged SeMNPV LEF-5 (MycSeLEF5), we constructed a plasmid containing the 280 bp *Opie2* promoter from OpMNPV, driving expression of an 873 bp ORF encoding a cMyc-Selef5 fusion (*mycSelef5*). The resulting plasmid, pBS-Opie2P-MycSelef5-neo (Fig. 2A), contains the *mycSelef5* fusion gene under the control of the *Opie2* promoter and the OpMNPV *gp64* promoter driving a *neo* gene. To analyze subcellular localization of AcMNPV LEF-5 (AcLEF5), we assembled constructs expressing either cMyc-tagged or EGFP-tagged AcMNPV LEF-5 (MycAcLEF5 and EGFPAcLEF5, respectively). The MycAcLEF5 expression plasmid, pPB-Opie1P-MycAclef5-neo, contains 591 bp of the *Opie1* promoter (from −557 to +34) from OpMNPV (Theilmann and Stewart, 1991) and an 831 bp ORF encoding a cMyc – AcMNPV *lef-5 (mycAclef5)* fusion. The EGFPAcLEF5 construct, pPB-Opie1P-EGFPAclef5-neo (Fig. 2A), contains the same *Opie1* promoter described above, and the 1521 bp *egfp* ORF fused with the AcMNPV *lef-5* ORF at amino acid 1. The same *neo* gene (described above) under the control of the *Opgp64* promoter was included in both constructs expressing cMyc-tagged AcMNPV LEF-5 and EGFP-tagged AcMNPV LEF-5 (pPB-Opie1P-MycAclef5-neo and pPB-Opie1P-EGFPAclef5-neo respectively). A control plasmid expressing EGFP (pPB-Opie1P-EGFP-neo) was also generated. This plasmid contains the *egfp* ORF under the control of the *Opie1* promoter, and the *neo* gene under the Opgp64 promoter. All of the plasmids described above were confirmed by restriction digestion and sequencing, and the sequence of each is available upon request.

Generation of AcMNPV lef-5 knockout and repaired viruses

To generate an AcMNPV virus containing a deleted *lef-5* gene, we used a long-primer PCR technique to delete the *lef-5* gene from a bacmid (bMON14272) containing the AcMNPV genome. Nucleotide sequence numbers for the AcMNPV genome refer to genbank accession and version numbers L22858.1 and GI:510708, respectively. To construct the *lef-5* knockout bacmid (bAc^{lef5ko}), a DNA fragment that contained a late-promoter driven reporter gene (*p6.9-GUS*) and a selectable marker cassette (*cat*, chloramphenicol acetyltransferase) was used to replace the *lef-5* ORF as illustrated in Fig. 1. The PCR fragment was amplified using long-primer PCR and Extensor High Fidelity PCR Master Mix (ABgene) to amplify the reporter gene/*cat* cassette from plasmid pKD3 + polyA/GUS (see supplementary data for cassette sequence and primers) and the Wt *lef-5* gene was replaced in bMON14272 using lambda RED recombinase as previously described (Datsenko and Wanner, 2000; Yamagishi et al., 2007). Insertion of the *gus-cat* cassette in bacmid bAc^{lef5ko} was verified by PCR analysis and by sequencing. To rescue the bacmid containing a *lef-5* knockout, we generated *lef-5* repair bacmids by reinserting *lef-5* into the polyhedrin locus. The *lef-5* repair bacmids were generated from pFastBac plasmid vectors containing *mycAclef5*, *egfpAclef5* or *Selef5egfp* genes. An *Opie1P-mycAclef5* cassette was excised from plasmid pPB-Opie1P-mycAclef5-neo as an EcoRI/XbaI fragment, which was subsequently blunted by Klenow fill-in, then ligated with SmaI-digested pBluescript-sk to create plasmid pBS-Opie1P-mycAclef5-SmaI. Then an *Opie1P-mycAclef5* fragment was excised with XbaI and HindIII from pBS-Opie1P-mycAclef5-SmaI and cloned into XbaI/HindIII digested pΔFBgus(R) vector (Lung et al., 2002). The resulting plasmid was named as pFB-mycAclef5 (Fig. 1B). Two more pFastBac transfer vectors, pFB-EGFPAclef5 and pFB-Selef5EGFP, were also constructed by following the same procedure as that used for creating pFB-mycAclef5. The *myc*- or *egfp*-tagged *lef-5* repair bacmids were generated by moving the tagged *Aclef-5* or *Selef-5* gene from pFB-mycAclef5, pFB-EGFPAclef5 or pFB-Selef5EGFP into the *polyhedrin* locus of bAc^{lef5ko} by transposition, according to standard methods (Luckow et al., 1993). Transformation and selection for the *lef5*-repair bacmids were performed as described by Lin and Blissard (Lin and Blissard, 2002a). The three *lef5*-repair bacmids were designated as bAc$^{lef5ko/FB\text{-}mycAclef5}$, bAc$^{lef5ko/FB\text{-}EGFPAclef5}$, and bAc$^{lef5ko/FB\text{-}Selef5EGFP}$ respectively (known elsewhere as bAc$^{lef5ko/FB\text{-}Opie1P\text{-}mycAclef5}$, bAc$^{lef5ko/FB\text{-}Opie1P\text{-}EGFPAclef5}$, and bAc$^{lef5ko/FB\text{-}Opie1P\text{-}Selef5EGFP}$). An empty pFastBac transfer vector pFB,

which contained no *lef-5* gene, was used to generate a control bacmid designated as bAc$^{lef5ko/FB}$ (Fig. 1B) (known as bAc$^{lef5ko/FB\text{-}Opie1P}$ elsewhere). The three *lef-5* repair bacmids and the control bacmids were confirmed by PCR analysis. An additional control virus, vAc$^{Wt/FB\text{-}mycEGFP}$, was constructed by inserting a cassette expressing a cMyc-tagged EGFP construct under the control of an OpMNPV *ie1* promoter (*Opie1P-mycEGFP*), and GUS under the control of the late *p6.9* promoter (*p6.9-GUS*), into the *polyhedrin* locus of the AcMNPV bacmid (bMON14272), by transposition.

Cell culture and transfection

Spodoptera frugiperda Sf9 cells were cultured in TNMFH complete medium containing 10% fetal bovine serum at 27 °C (Hink, 1970; O'Reilly et al., 1992; Summers and Smith, 1987). Transfection of Sf9 cells with plasmids or bacmids was carried out essentially as described previously (Mangor et al., 2001).

Generation of stable SeLEF5EGFP-expressing cell lines

For generating stable cell lines expressing SeLEF5EGFP, Sf9 cells were plated at a density of 1×10^6 cells per well (34-mm diameter wells). The cells were transfected with 2 μg of plasmid pBS-Opie2P-Selef5EGFP-neo as described previously (Zhou and Blissard, 2008a). The pBS-Opie2P-Selef5EGFP-neo plasmid contains a neomycin phosphotransferase gene under the control of an OpMNPV *gp64* promoter and a *Selef5-egfp* fusion gene under the control of an OpMNPV *ie2* promoter (a highly active early promoter in Sf9 cells) (Pfeifer et al., 1997). At 48 h post transfection, cells were replated at low density (app. 5×10^5 cells per flask in 25-cm^2 flasks) and placed in TNMFH complete medium containing G418 (0.8 mg/ml). Cells were subcultured every 3 days for 12 days. During this period, the mock-transfected Sf9 control cells died. The stably transfected cells that were G418-resistant were replated in TNMFH complete medium and allowed to recover for 24 h. The G418-resistant cells were diluted to a density of approximately one cell per 100 μl in TNMFH complete medium and plated at 100 μl per well in 96-well plates. Wells containing single cells were scored on the same day or the following morning. Single cell-derived colonies were grown for approximately 2–3 weeks, and then transferred to 24-well or 12-well plates. After 7–10 days, the single cell derived cell lines were replated in 6-well plates or 25-cm^2 flasks and EGFP fluorescence was used to identify and assess SeLEF5EGFP-expressing cell lines. Selected cell lines were then incubated again in TNMFH medium containing G418 for an additional 6 days. The independently cloned cell lines were subsequently verified by Southern blot analysis. In order to develop cell lines with more uniform high-level expression of the LEF-5 construct, a second single-cell cloning step was performed using the primary cell lines.

Southern blot analysis

Genomic DNA was extracted from stably transfected cell lines, or from non-transfected Sf9 cells according to established procedures (van Oers et al., 1999). Ten microgram of genomic DNA was digested with HindIII and separated in a 0.8% agarose gel. A digoxigenin (DIG)-labeled 0.8-kb *Selef-5* coding sequence was used as a probe (Fig. 2, Probe). Gel preparation, hybridization, and washing were performed according to the standard protocol (Sambrook et al., 1989) and the instruction manual supplied with the DIG-labeling and hybridization kit (Roche Applied Science).

Western-blot analysis

To examine proteins by Western blot analysis, we extracted EGFPAcLEF5 and SeLEF5EGFP fusion proteins from the nuclear fractions of Sf9 cells infected with repair virus (vAc$^{lef5ko/FB\text{-}EGFPAclef5}$ or vAc$^{lef5ko/FB\text{-}Selef5EGFP}$) at 48 h p.i. (MOI 5) as previously described (Jarvis et al., 1991; Murges et al., 2001). The fusion protein MycAcLEF5 was extracted from vAc$^{lef5ko/FB\text{-}mycAclef5}$-infected Sf9 cells with RIPA buffer (50 mM Tris–HCl, pH 8.0, 150 mM NaCl, 0.1% SDS, 0.5% Na Deoxycholate, 1% Triton X-100 plus 0.5 mM PMSF and proteinase inhibitor cocktail, Roche). EGFP and MycAcGP64 (Zhou and Blissard, 2008b) were used as positive controls. The protein extract collected from the control virus, vAc$^{lef5ko/FB}$, and Wt-AcMNPV-infected Sf9 cells was used as a negative control. Western blot analysis was performed as described previously (Zhou and Blissard, 2008b). Briefly, 15 μl of cell lysate or nuclear extract was mixed with 5x SDS-PAGE loading buffer and boiled for 5 min prior to analysis by 12% SDS-PAGE. Proteins were transferred to Immobilon-P membranes (Millipore) and hybridized with anti-GFP polyclonal antibody at a dilution of 1:1000 (Invitrogen) or with anti-myc monoclonal antibody at a dilution of 1:75 (ATCC CRL-1729; Myc 1-9E10.2). Immunoreactive proteins were detected using alkaline phosphatase-conjugated anti-mouse or anti-rabbit IgG antibody and NBT/BCIP (Promega).

GUS assays

To identify cells expressing beta-glucuronidase from the GUS reporter gene, bacmid-transfected or virus-infected Sf9 cells were incubated in an X-Gluc solution (1 mg/ml 5-Bromo-4-chloro-3-indoxyl-beta-D-glucuronide, 5 mM potassium ferrocyanide, 5 mM potassium ferricyanide, 50 mM Na_3PO4, pH 7.0, Gold Biotechnology Co.) for 4 h at 27 °C after removing media. GUS positive cells were recorded by visible light microscopy.

Analysis of viral replication

To analyze viral replication of vAc^{lef5ko}, vAc^{lef5ko} virus particles were generated by infection of the SeLEF5EGFP-expressing cell line, then titered in the same line and used to infect both Sf9 cells and SeLEF5EGFP-expressing cells. For virus growth curves, Sf9 cells or SeLEF5EGFP-expressing Sf9 cells (5×10^5 cells per well) were infected in triplicate with each virus (vAc^{lef5ko}, a repair virus vAc$^{lef5ko/FB\text{-}mycAclef5}$, and a virus containing a WT *lef-5* locus vAc$^{Wt/FB\text{-}mycEGFP}$) at an MOI of 5. After a 1 h incubation, cells were washed twice and the medium was replaced with fresh TNMFH medium. Supernatants were collected at the indicated times (0, 12, 24, 48, 72, 96 and 168 h p.i.) and the titers of all supernatants were determined by a $TCID_{50}$ end point dilution assay on Sf9 cells or SeLEF5EGFP-expressing Sf9 cells (O'Reilly et al., 1992).

Analysis of viral transcription by qPCR

For analysis of viral transcripts by reverse transcription qPCR (RT-qPCR), Sf9 cells (1×10^6 cells per well) were infected in triplicate (MOI 2) with vAc^{lef5ko} (generated in the SeLEF5EGFP-expressing cell line) and a control virus containing a wild type *lef-5* locus and a myc-tagged EGFP marker (vAc$^{Wt/FB\text{-}mycEGFP}$). Total RNA was extracted from infected cells at 48 h p.i. with an RNeasy Mini Kit (QIAGEN). First-strand cDNA synthesis was primed with gene-specific primers using the ProtoScript M-MuLV First Strand cDNA Synthesis Kit (New England BioLabs). RNA extracted from uninfected cells was used to determine the background level for each gene and primer set. Primers for RT-qPCR were selected from the non-overlapping region of the target genes and are listed in Table 1. RNAs isolated for transcript analysis were treated with DNase I prior to RT-PCR amplification. Parallel samples that were not treated with DNaseI were used to determine the viral DNA genome copy number and to normalize transcript levels relative to viral genome copy number. The transcript level of each gene was normalized to the viral genomic copy number (see supplemental data). Transcript copy numbers were determined using 800 pg of total RNA as the RT-qPCR template. An equivalent volume was used as template for analysis of viral DNA. For

comparison of the effects of the *lef-5* knockout on each transcript, the transcript level determined from the control virus ($vAc^{Wt/FB\text{-}mycEGFP}$) was assigned a value of 1. The normalized transcript levels were calculated as transcript copies per thousand viral genomes. The statistical analysis of transcript data was performed by the Student's *t*-test. Comparisons are presented as percentages relative to control virus $vAc^{Wt/FB\text{-}mycEGFP}$ and direct transcript data are included as supplemenal data (Table S-1).

RT-qPCR was performed as follows. For each reaction 13 μl of a qPCR master mix (containing 7.5 μl of SYBR GreenER qPCR SuperMix (Invitrogen) and adjusted to 0.2 M of primer with water), was added to 2 μl of template. Templates consisted of either 1st-strand cDNA (generated from 800 pg of total RNA previously treated with DNase I) or 800 pg of total nucleic acids (RNA + DNA, with no DNAase treatment). Standard curves were generated with six serial tenfold dilutions for each amplicon, ranging from 10^1 to 10^6 copies of the PCR fragment-containing plasmids. qPCR was performed on an ABI 7900 Real-Time PCR System with the following reaction parameters: 95 °C for 10 min then 40 cycles of 95 °C for 15 s, 60 °C for 1 min.

Analysis of viral DNA replication by qPCR

To analyze viral DNA replication by qPCR, Sf9 cells were infected in triplicate with viruses vAc^{lef5ko}, $vAc^{lef5ko/FB\text{-}mycAclef5}$, and $vAc^{Wt/FB\text{-}mycEGFP}$ (5×10^5 cells per well, MOI 5). Total DNA was extracted at 0, 12, 24, 36, 48, and 72 h p.i., using a DNeasy Blood & Tissue kit (QIAGEN) according to the manufacture's protocol. DNA from uninfected Sf9 cells was used as a control template and qPCR was performed as described above. The *odv-e56* primer set (Table 1) was used for analysis of viral DNA replication. Each reaction included 100 pg of total DNA.

Immunofluorescence assays and confocal microscopy

To examine the sub-cellular localization of AcMNPV LEF-5 or SeMNPV LEF-5, Sf9 cells (approximately 1×10^6 cells grown on cover slips) were transfected with plasmid pPB-Opie1P-EGFPAclef5-neo or plasmid pBS-Opie2P-Selef5EGFP-neo, respectively. For comparative analysis of localization of cMyc-tagged LEF-5 proteins with EGFP-tagged LEF-5 proteins, cells were cotransfected with plasmids pPB-Opie1P-MycAclef5-neo and pPB-Opie1P-EGFPAclef5-neo (expressing cMyc-tagged AcMNPV LEF-5 and EGFP-tagged AcMNPV LEF-5, respectively); or with plasmids pBS-Opie2P-MycSelef5-neo and pBS-Opie2P-Selef5EGFP-neo (expressing cMyc-tagged SeMNPV LEF-5 and EGFP-tagged SeMNPV LEF-5, respectively). At 48 h p.t., Sf9 cells were fixed with methanol:acetone (1:1) at −20 °C for 10 min. MycSeLEF5 was detected by immunofluorescence with an anti-myc MAb (ATCC CRL-1729; Myc 1-9E10.2) and an Alexa Fluor 594 goat anti-mouse IgG (Invitrogen). For co-infections of Sf9 cells, two viruses ($vAc^{lef5ko/FB\text{-}Selef5EGFP}$ and $vAc^{lef5ko/FB\text{-}mycAclef5}$, or $vAc^{lef5ko/FB\text{-}EGFPAclef5}$ and $vAc^{lef5ko/FB\text{-}mycAclef5}$) were used to infect 2×10^6 Sf9 cells at an MOI of 2. At 48 h p.i., infected cells were fixed as described above. The MycAcLEF5 was detected by immunofluorescence assays with an anti-myc MAb as described above. To analyze colocalization of EGFPAcLEF5 with AcIE1, Sf9 cells were infected with virus $vAc^{lef5ko/FB\text{-}EGFPAclef5}$ (MOI 5) at 27 °C for 1 h, then washed once with TNMFH medium and cultured in TNMFH medium at 27° for various periods. The time post infection was calculated from the point that viral inoculum was added. Cells were fixed with cold methanol at −20 °C for 10 min. AcMNPV IE-1 was detected by immunofluorescence with an anti-IE-1 MAb "IE1-4B7" (Ross and Guarino, 1997) and Alexa Fluor 594 conjugated goat anti-mouse IgG (Invitrogen), as described previously (Lin and Blissard, 2002a). Fluorescence was detected on a LEICA TCS-SP5 confocal microscope system.

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